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Menin Critically Links MLL Proteins with LEDGF on Cancer-Associated Target Genes

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SUMMARY

Menin displays the unique ability to either promote oncogenic function in the hematopoietic lineage or suppress tumorigenesis in the endocrine lineage; however, its molecular mechanism of action has not been defined. We demonstrate here that these discordant functions are unified by menin's ability to serve as a molecular adaptor that physically links the MLL (mixed-lineage leukemia) histone methyltransferase with LEDGF (lens epithelium-derived growth factor), a chromatin-associated protein previously implicated in leukemia, autoimmunity, and HIV-1 pathogenesis. LEDGF is required for both MLL-dependent transcription and leukemic transformation. Conversely, a subset of menin mutations in multiple endocrine neoplasia type 1 patients abrogate interaction with LEDGF while preserving MLL interaction but nevertheless compromise MLL/menin-dependent functions. Thus, LEDGF critically associates with MLL and menin at the nexus of transcriptional pathways that are recurrently targeted in diverse diseases.

INTRODUCTION

Cancer results from genetic and epigenetic perturbations that cause the unbalanced actions of oncoproteins and tumor suppressors. The menin tumor suppressor is implicated in cancer pathogenesis and transcriptional regulation as an integral component of the MLL (mixed-lineage leukemia) histone methyltransferase (HMT) complex, which promotes specific trimethylation of lysine 4 on histone H3, an epigenetic mark associated with transcriptionally active chromatin (Hughes et al., 2004; Milne et al., 2002; Nakamura et al., 2002; Yokoyama et al., 2004). Menin is a product of the *MEN1* gene, whose loss of function causes the human cancer syndrome known as multiple endocrine neoplasia type 1 (MEN1) (Chandrasekharappa et al., 1997). It is also implicated in the dynamic regulation of pancreatic β cell proliferation in response to normal physiologic demands during pregnancy whose failure may promote gestational diabetes (Karnik et al., 2007). These normal and pathologic roles in endocrine cells reflect specific requirements for the MLL/menin HMT complex to maintain expression of the *CDKN1B* and *CDKN2C* genes, which encode the cyclin-dependent kinase inhibitors (CDKIs) $p27^{KIP1}$ and $p18^{Ink4c}$, respectively (Karnik et al., 2005; Milne et al., 2005). Their compromised expression following mutation or deletion of *MEN1* leads to hyperproliferation of endocrine lineage cells (Bertolino et al., 2003; Franklin et al., 1998; Crabtree et al., 2001). Many mutations of menin in endocrine tumors abrogate its ability to associate with the MLL HMT complex (Hughes et al., 2004); however, the specific molecular function of menin is currently unknown.

Transcriptional regulation of *HOX* genes is also exquisitely dependent on MLL, which is required to establish the embryonic body plan during development (Yu et al., 1995) and to promote progenitor expansion and stem cell self-renewal in the hematopoietic lineage (Jude et al., 2007; McMahon et al., 2007). In a subset of acute leukemias, chromosomal aberrations generate chimeric MLL oncoproteins that cause constitutive expression of *HOX* genes, a key feature of MLL leukemia pathogenesis (Daser and Rabbitts, 2004; Hess, 2004). In contrast to its tumor suppressor role in MEN1 tumorigenesis, menin serves as an

SIGNIFICANCE

Menin is a tumor suppressor whose loss of function causes the human cancer syndrome known as multiple endocrine neoplasia type 1. Conversely, menin serves as an essential oncogenic cofactor for MLL (mixed-lineage leukemia) oncoproteins in leukemic transformation. These discordant functions are attributed in part to tissue-specific differences in critical MLL target genes; however, the molecular role of menin in these contrasting oncogenic settings remains undefined. We demonstrate here that menin serves as a molecular adaptor that tethers the MLL histone methyltransferase and its oncogenic counterparts to LEDGF (lens epithelium-derived growth factor), a transcriptional coactivator previously implicated in cancer, autoimmunity, and HIV proviral integration. A requirement for LEDGF in MLL/menin transcriptional and oncogenic actions places it at the center of a key epigenetic pathway in cancer pathogenesis.

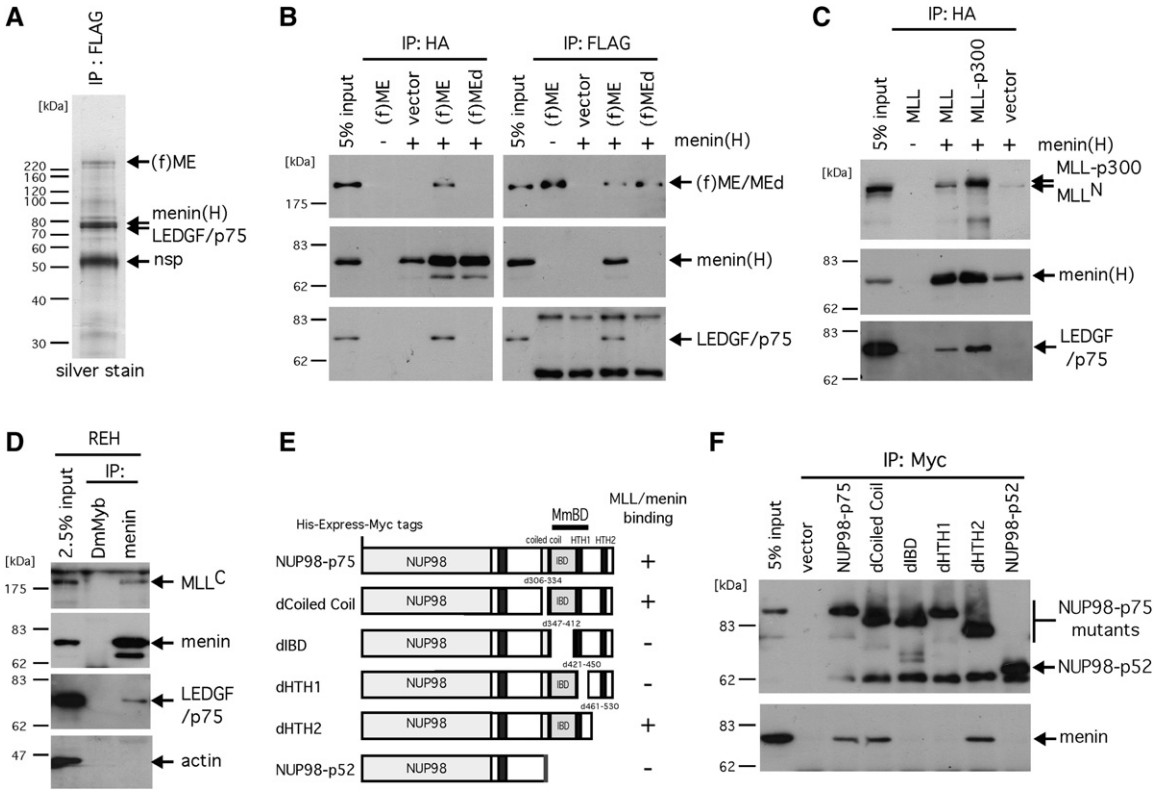

Figure 1. LEDGF and NUP98-LEDGF Associate with the MLL/Menin Histone Methyltransferase Complex

(A) Silver stain image of an SDS-PAGE analysis shows the presence of LEDGF in the purified MLL-ENL/menin immunoprecipitate. FLAG-tagged MLL-ENL ((f)ME) and HA-tagged menin (menin(H)) were transiently expressed in large-scale culture of 293T cells. A nuclear extract was prepared from the cells and subjected to immunopurification using anti-FLAG antibody. Protein bands from SDS-PAGE analysis were analyzed by mass spectrometry and identified as indicated by arrows. The band at 52 kDa is a nonspecific (nsp) product that was also observed in the control immunoprecipitate of a nuclear extract from nontransduced cells (not shown). Protein standards are shown on the left.

(B) Reciprocal immunoprecipitations (IPs) of the MLL-ENL/menin complex. (f)ME or a mutant ((f)MEd) lacking the high-affinity menin-binding motif were transiently expressed in 293T cells with (+) or without (–) HA-tagged menin. Nuclear extracts from the transfected cells were subjected to IP with anti-FLAG or anti-HA antibodies followed by immunoblotting with anti-FLAG, anti-HA, and anti-LEDGF (p75) antibodies.

(C) MLL or MLL-p300 was transiently expressed with (+) or without (–) HA-tagged menin in 293T cells and subjected to IP with anti-HA antibody followed by immunoblotting with anti-MLL^N, anti-HA, and anti-LEDGF antibodies.

(D) To detect endogenous MLL/menin/LEDGF association, nuclear extract of REH cells was subjected to IP with anti-menin antibody followed by immunoblotting with anti-MLL^C, anti-menin, anti-LEDGF, and anti-actin antibodies. Anti-*Drosophila* Myb (DmMyb) antibody was used as a negative control.

(E) Schematic representations of the NUP98-LEDGF mutants are shown with a summary of their binding properties with MLL/menin on the right. The identified minimum MLL/menin-binding domain (MmBD; residues 335–460) is indicated.

(F) His-Express-Myc-tagged NUP98-LEDGF proteins were expressed in 293T cells and subjected to IP with anti-Myc antibody followed by immunoblotting with anti-Express epitope and anti-menin antibodies.

essential cofactor for MLL oncoproteins to sustain *HOX* gene misexpression and maintain leukemic transformation (Caslini et al., 2007; Chen et al., 2006; Yokoyama et al., 2005). Thus, menin has the unusual ability to either promote oncogenic function in the hematopoietic lineage or suppress tumorigenesis in the endocrine lineage. These discordant functions are attributed to tissue-specific differences in critical target genes that encode oncogenic versus tumor suppressor proteins in hematopoietic versus endocrine cells, respectively. However, a unifying molecular role for menin underlying transcriptional regulation by normal and oncogenic MLL proteins has not been defined.

Here we demonstrate that menin tethers MLL with the p75 isoform of LEDGF (lens epithelium-derived growth factor, also called DFS70/p75/PSIP1), a chromatin-associated protein that is also targeted in various diseases including cancer (Ahuja et al., 2000; Daugaard et al., 2007; Huang et al., 2007), autoimmunity (Ganapathy and Casiano, 2004), and AIDS (Ciuffi and Bushman, 2006), thereby providing a crucial molecular link with chromatin for an epigenetic complex at the center of multiple pathologic processes.

RESULTS

Wild-Type and Oncogenic MLL/Menin Protein Complexes Specifically Associate with LEDGF

Menin is essential for MLL-dependent transcriptional regulation and leukemic transformation (Caslini et al., 2007; Chen et al., 2006; Yokoyama et al., 2005); however, its molecular mechanism of action has not been defined. We hypothesized that menin might further recruit an unknown factor to the MLL/menin

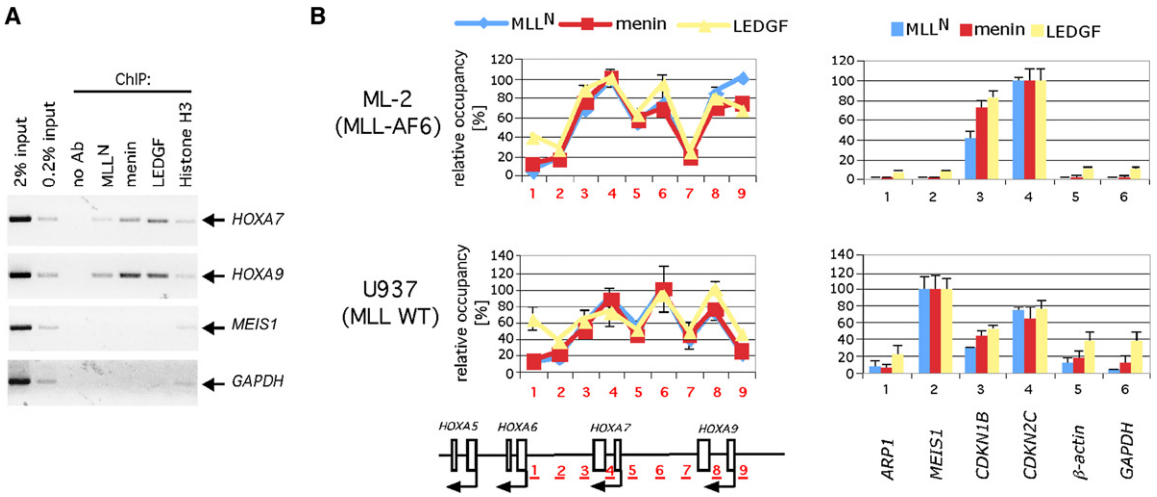

Figure 2. MLL Proteins Colocalize with LEDGF on Chromatin of Cancer-Associated Genes

(A) Chromatin immunoprecipitation (ChIP) was performed on ML-2 cells using anti-MLL^N, anti-menin, and anti-LEDGF antibodies. MLL-AF6, menin, and LEDGF occupancy was specifically observed at the *HOXA7* and *HOXA9* loci, but not on the *MEIS1* and *GAPDH* loci. Negative and positive controls consisted of no antibody and anti-histone H3 antibody, respectively.

(B) ChIP followed by quantitative PCR was performed on ML-2 and U937 cells using anti-MLL^N, anti-menin, and anti-LEDGF antibodies. Values are expressed relative to the maximum value (arbitrarily set at 100%) in each group, with error bars representing standard deviations for triplicate PCR analyses.

complex. Since stably associated factors of MLL have been extensively characterized (Dou et al., 2005; Yokoyama et al., 2004), we inferred that potential novel factors might associate more weakly. Thus, the MLL-ENL/menin complex was biochemically purified by one-step affinity purification from cells transiently expressing high levels of the respective proteins to minimize the loss of weakly associated proteins during the purification procedure. In the purified materials, we identified a 75 kDa nuclear protein termed LEDGF (Figure 1A) previously implicated in transcriptional coactivation (Ge et al., 1998) and lentiviral integration (Ciuffi and Bushman, 2006).

Specific association of LEDGF with the MLL-ENL/menin complex was confirmed by reciprocal immunoprecipitation (IP) experiments using anti-FLAG or anti-HA antibodies to pull down MLL-ENL or menin, respectively, which showed that endogenous LEDGF consistently coprecipitated with transiently expressed menin and MLL-ENL (Figure 1B). However, expression of menin or MLL-ENL alone did not result in coimmunoprecipitation of LEDGF at a detectable level. Consistent with these observations, a mutant MLL-ENL (MEd) that lacks the high-affinity menin-binding motif (hMBM) and therefore does not interact with menin efficiently (Yokoyama et al., 2005) failed to coprecipitate with endogenous LEDGF despite the presence of abundant menin (Figure 1B). These data demonstrate that LEDGF associates conjointly with MLL-ENL and menin, but not with either protein separately.

LEDGF also coprecipitated with menin in association with transiently expressed wild-type MLL and other MLL fusion proteins (Figure 1C; see also Figure S1A available online). At a very low level, LEDGF was detected in the endogenous MLL/menin complex in REH cells as well (Figure 1D). Furthermore, the NUP98-LEDGF fusion protein created by chromosomal translocation in acute leukemia (Ahuja et al., 2000) also coprecipitated with the MLL/menin complex, suggesting a possible pathogenic association. Interaction was dependent on the HIV-1 integrase-binding domain (IBD) (Cherepanov et al., 2004) as revealed by deletion mutagenesis (Figures 1E and 1F), consistent with the inability of a naturally occurring isoform of LEDGF (p52) that lacks the IBD to coimmunoprecipitate with MLL/menin (Figure S1B). Thus, the p75 isoform of LEDGF is a specific cofactor for both wild-type and oncogenic MLL/menin protein complexes.

LEDGF Colocalizes with MLL Proteins and Menin on Crucial Target Genes

Chromatin immunoprecipitation (ChIP) analyses demonstrated that LEDGF colocalizes with MLL/menin complexes at target sites within *HOXA*, *MEIS1*, and *CDKI* genes (Figure 2; Figure S2), all previously linked with MLL/menin pathologies (Ayton and Cleary, 2003; Karnik et al., 2005; Milne et al., 2005; Wong et al., 2007). This was the case in U937 cells, which lack *MLL* gene rearrangement and express only wild-type MLL (Dreyling et al., 1996; Guenther et al., 2005), and in ML-2 cells, which exclusively express MLL-AF6 in the absence of wild-type MLL (Tanabe et al., 1996; Yokoyama et al., 2005). The occupancy of MLL-AF6 or wild-type MLL throughout the *HOXA* locus and other target loci extensively overlapped with menin and LEDGF (Figure 2B). Thus, LEDGF forms stable complexes with MLL (or MLL oncoproteins) in vivo on genes implicated to critically mediate MLL/menin-associated biology and disease.

LEDGF Is Required for Initiation of Leukemic Transformation by MLL Oncoproteins

A structure/function analysis of MLL-ENL revealed that deletions spanning MLL residues 112–153 completely abolished LEDGF binding while preserving menin association (Figures 3A and 3B; Figure S3). This defined an evolutionally conserved region within the amino-terminal portion of MLL as a specific LEDGF-binding domain (LBD) distinct from the hMBM.

The MLL-ENL deletion mutants were evaluated for oncogenic activity using a transformation assay that reads out the ability of *MLL* fusion genes to induce enhanced self-renewal of myeloid

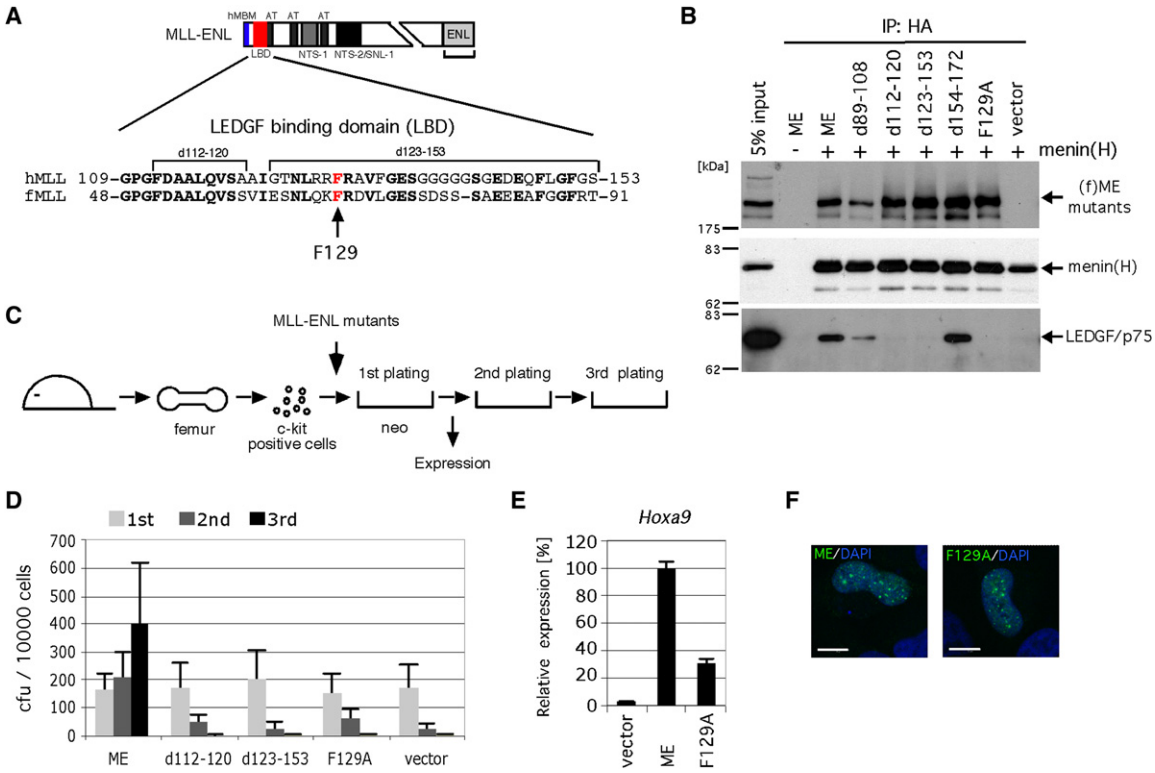

Figure 3. MLL Oncoproteins Associate with LEDGF to Initiate Myeloid Transformation

(A) Schematic structure of the MLL-ENL oncoprotein. The amino acid sequence of MLL encompassing the LEDGF-binding domain (LBD; residues 109–153 denoted by red box) is aligned with that of fugu MLL. Arrow indicates the phenylalanine residue converted to alanine.

(B) Various mutants of (f)ME were transiently expressed with (+) or without (–) HA-tagged menin in 293T cells and subjected to IP with anti-HA antibody followed by immunoblotting with anti-FLAG, anti-HA, and anti-LEDGF antibodies.

(C) Experimental scheme for the myeloid progenitor transformation assay. The time point at which *Hoxa9* expression was measured (end of 1st plating) is indicated.

(D) Colony-forming units (cfu) per 10^4 plated cells are shown for each round of plating. Error bars represent standard deviations of three independent experiments.

(E) Relative expression levels of *Hoxa9* are shown for 1st-round colonies. Expression levels are normalized to β-*actin* and expressed relative to the ME value (arbitrarily set at 100%). Error bars represent standard deviations of triplicate PCR analyses.

(F) Subnuclear localizations of MLL fusion proteins in HeLa cells are shown as a merged image of signals for FITC (MLL) and DAPI (DNA). Scale bar = 10 μm.

progenitors in vitro compared with control cells, which rapidly exhaust their clonogenic potentials. All deletion mutants unable to associate with LEDGF were also unable to transform myeloid progenitors (Figures 3C and 3D). Moreover, a single amino acid substitution within the LBD that converted an evolutionally conserved phenylalanine to alanine (F129A) completely abolished LEDGF binding and myeloid transformation. The F129A mutant was also transcriptionally incapable of maintaining *Hoxa9* expression in transduced myeloid progenitors (Figure 3E) despite efficient expression and localization in nuclear bodies (Figure 3F). Thus, specific association with LEDGF is essential for misregulation of *Hoxa9* expression and transformation of myeloid progenitors by the MLL oncoprotein.

Menin Is an Adaptor that Tethers MLL Oncoproteins with LEDGF in Transformed Myeloid Progenitors

LEDGF contains a highly conserved motif (PWWP) that is structurally related to the so-called "royal family" of domains present in a wide variety of chromatin-associated proteins and implicated in recognition of modified nucleosomes (Maurer-Stroh et al., 2003). The PWWP domain of LEDGF is necessary to associate with chromatinized DNA and target HIV-1 genome integration to transcriptionally active sites (Botbol et al., 2008; Shun et al., 2007). We hypothesized, therefore, that menin's role is to tether MLL proteins with LEDGF as an adaptor, which in turn promotes association of the complex with transcriptionally active chromatin through its PWWP domain.

To test this hypothesis, MLL oncoproteins were engineered to circumvent menin by replacing the hMBM with the PWWP domain of LEDGF. The resultant modified MLL-ENL (pME) was no longer capable of stably associating with menin and LEDGF (Figures 4A and 4B) but nevertheless strongly upregulated *Hoxa9* expression and efficiently transformed myeloid progenitors (Figures 4A and 4C). Consistent with these observations, pME function was not compromised by F129A mutation of the LBD, which otherwise abrogates noncovalent LEDGF interaction. Fusion with PWWP, however, did not bypass the requirement for the MLL fusion partner moiety in myeloid transformation by pME or a similarly engineered MLL-AF10 (Figures 4A and 4B). ChIP analysis of pME-transformed cells showed that pME occupied the *Hoxa9* locus in the absence of menin and LEDGF (Figure 4E). Mutation of an evolutionally conserved tryptophan

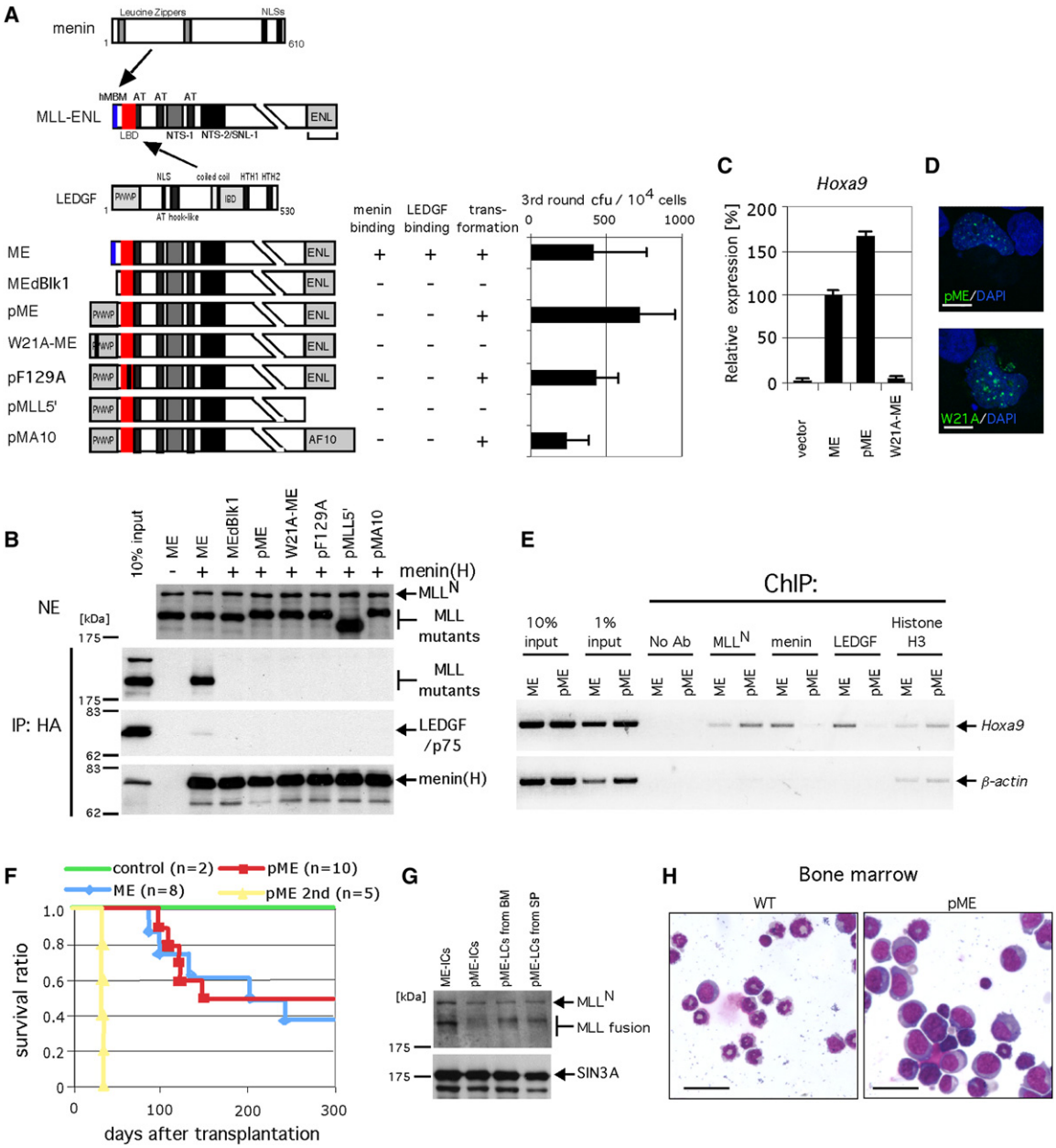

Figure 4. Menin Tethers LEDGF with MLL Oncoproteins

(A) Schematic structures of menin, MLL-ENL, and LEDGF are shown at the top, with sites of intermolecular interactions indicated by arrows. Binding properties and transforming abilities are summarized to the right of the respective MLL-ENL constructs. The numbers of 3rd-round cfu in myeloid progenitor assays are shown at right, with error bars representing the standard deviations of three independent experiments.

(B) Various mutants of pME were transiently expressed with (+) or without (–) HA-tagged menin in 293T cells and subjected to IP with anti-HA antibody followed by immunoblotting with anti-MLL^N, anti-HA, and anti-LEDGF antibodies. Upper panel shows immunoblot of the nuclear extracts (NE) to determine protein inputs. Mutants lacking the high-affinity menin-binding motif failed to coimmunoprecipitate with menin and LEDGF.

(C) Relative expression levels are shown for *Hoxa9* in 1st-round colonies. Expression levels are normalized to β-*actin* and expressed relative to the ME value (arbitrarily set at 100%). Error bars represent standard deviations of triplicate PCR analyses.

(D) Subnuclear localization of the pME mutants in HeLa cells is shown as a merged image of signals for FITC (MLL) and DAPI (DNA). Scale bar = 10 μm.

(E) ChIP analysis of ME- or pME-transformed myeloid progenitors was performed using the antibodies indicated at the top. Amplicons upstream of the *Hoxa9* and β-*actin* genes were analyzed.

(F) Survival curves are shown for mice transplanted with the indicated transduced cells. The numbers of mice analyzed (n) are indicated.

(G) Expression of ME and pME proteins in immortalized cells (ICs) and leukemic cells (LCs) from bone marrow (BM) and spleen (SP) was analyzed by immunoblotting with anti-MLL^N and anti-SIN3A (control) antibodies.

(H) Morphology is shown for normal bone marrow (WT, left panel) and pME leukemic blasts (pME, right panel) following May-Grunwald/Giemsa staining of cytospin preparations. Scale bar = 15 μm.

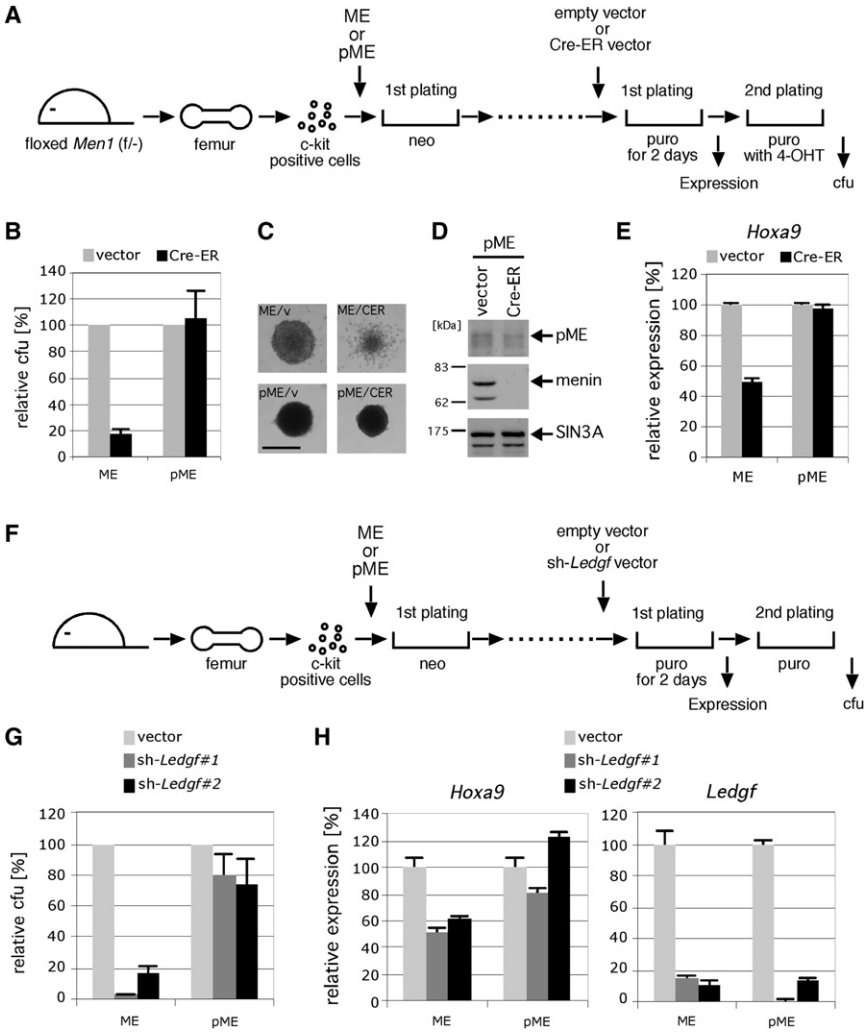

Figure 5. LEDGF Is Necessary for Maintenance of MLL Leukemic Transformation

(A) Experimental scheme for conditional inactivation of *Men1* in MLL-transformed cells. The time points at which cfu or gene expression was measured are indicated.

(B) Relative cfu for cells transformed by ME or pME in the absence (vector) or presence (Cre-ER) of *Men1* inactivation (vector controls are arbitrarily set at 100%). Error bars represent standard deviations of three independent analyses.

(C) Morphologies for representative colonies from the experiment in (B). Scale bar = 150 μm.

(D) Western blot showing expression of pME, menin, and SIN3A proteins after *Men1* inactivation.

(E) Relative expression levels of *Hoxa9* are shown for 1st-round colonies after Cre-ER transduction. Expression levels were normalized to β-*actin* and expressed relative to the vector control values (arbitrarily set at 100%). Error bars represent standard deviations of triplicate PCR analyses.

(F) Experimental scheme for conditional inactivation of *Ledgf* by shRNA-mediated knockdown. The time points at which cfu or gene expression was measured are indicated.

(G) The relative cfu activity of ME- or pME-transformed cells is shown with (sh-*Ledgf#1* and sh-*Ledgf#2*) or without (vector) *Ledgf* knockdown (the vector controls are arbitrarily set at 100%). Error bars represent standard deviations of three independent analyses.

(H) Relative expression levels of *Hoxa9* and *Ledgf* are shown for 1st-round colonies after shRNA vector transduction. Expression levels are normalized to β-*actin* and expressed relative to the vector control values (arbitrarily set at 100%). Error bars represent standard deviations of triplicate PCR analyses.

to alanine (W21A) in the PWWP motif abolished both *Hoxa9* upregulation and myeloid transformation, confirming a critical role of the PWWP domain in pME function (Figures 4A, 4C, and 4D).

Transplantation of pME-transformed cells into congenic recipients induced acute leukemias with latencies and penetrance similar to those of ME-transplanted mice (Figures 4F and 4G). In both cohorts, blast cells with morphologic features characteristic of acute monocytic leukemia massively infiltrated the spleen, liver, and bone marrow (Figure 4H; Figure S4), and primary leukemia cells expressing pME were transplantable to secondary recipients, which developed leukemia with short latencies (Figures 4F and 4G). Thus, pME induces leukemia in vivo despite its inability to associate with menin.

A conditional knockout approach was employed to further confirm that menin is dispensable for leukemic transformation by pME. Myeloid progenitors harvested from *Men1* floxed mice were transformed by ME or pME and subsequently transduced with Cre-ER (Cre recombinase fused with estrogen receptor), which was conditionally activated in the presence of 4-hydroxytamoxifen (4-OHT) to inactivate the *Men1* gene (Figure 5A). While ME-transformed cells lost their clonogenicity and displayed predominantly differentiated colony morphologies after *Men1* inactivation as previously reported (Yokoyama et al., 2005), pME-transformed cells retained their transformed phenotype (Figures 5B and 5C) and maintained high-level *Hoxa9* expression in the absence of menin (Figures 5D and 5E). Thus, covalent fusion of the PWWP domain of LEDGF to MLL oncoproteins fully bypasses the requirement for menin in MLL-mediated leukemic transformation, indicating that the sole molecular requirement for menin as an oncogenic cofactor in MLL-associated leukemogenesis is to tether the PWWP domain of LEDGF to the MLL oncoprotein.

Sustained LEDGF Expression Is Required for Maintenance of Leukemic Transformation by MLL Oncoproteins

To assess whether LEDGF must be continuously present for maintenance of MLL-dependent transformation, its expression was knocked down in ME- and pME-transformed progenitors using shRNA techniques (Figure 5F). Efficient (>80%) knockdown of *Ledgf* expression obtained by two different shRNAs (Figure 5H) markedly reduced the clonogenic potential of ME-transformed cells, whereas pME-transformed cells were unaffected (Figure 5G). Furthermore, *Ledgf* knockdown impaired *Hoxa9* expression in ME-transformed cells, but not in

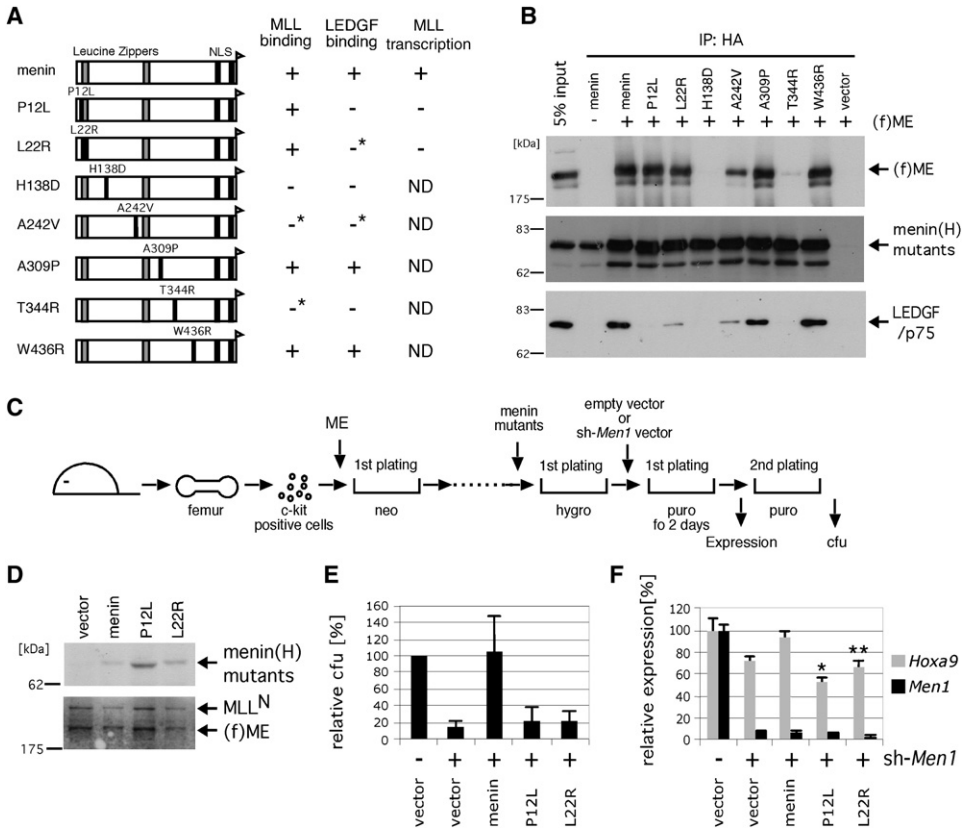

Figure 6. Menin Functionally Interacts with LEDGF

(A) Schematic structures of menin and its mutants. The abilities of each protein to associate with MLL or LEDGF and to rescue MLL-dependent transcription are summarized on the right. * indicates impaired association as opposed to complete loss of association. ND, not determined.

(B) Various HA-tagged menin proteins were transiently expressed with (+) or without (−) (f)ME in 293T cells and subjected to IP with anti-HA antibody followed by immunoblotting with anti-FLAG, anti-HA, and anti-LEDGF antibodies.

(C) Experimental scheme for rescue of *Men1* knockdown by wild-type or mutant menin proteins. The time points at which cfu or gene expression was measured are indicated.

(D) Western blot analysis showing expression of menin mutants in ME-transformed cells detected by anti-HA and anti-MLL^N antibodies, respectively.

(E) Relative cfu for ME-transformed cells transduced with various menin mutants with (+) or without (−) *Men1* knockdown (vector control is arbitrarily set at 100%). Error bars represent standard deviations of three independent analyses.

(F) Relative expression levels of *Hoxa9* and *Men1* for 1st-round colonies after *Men1* knockdown. Expression levels are normalized to *Gapdh* and expressed relative to the ME/vector value (arbitrarily set at 100%). Error bars represent standard deviations of triplicate PCR analyses. p values for differences compared to wild-type menin rescue were determined by unpaired t test. *$p < 0.0005$; **$p < 0.005$.

pME-transformed cells (Figure 5H). Thus, LEDGF is required to maintain aberrant *Hox* gene expression and transformation induced by MLL oncoproteins.

Implication of LEDGF in Menin Tumor Suppression

To investigate the role of LEDGF in MEN1 tumorigenesis, menin proteins harboring point mutations found in MEN1 patients were analyzed for their abilities to associate with MLL proteins and LEDGF. Several menin mutants (H138D, A242V, and T344R) displayed no or markedly reduced interactions with MLL (Figures 6A and 6B), consistent with their previously demonstrated inability to coimmunoprecipitate with HMT activity (Hughes et al., 2004). They also failed to associate with LEDGF, compatible with our foregoing results (Figure 1B) indicating that LEDGF interacts conjointly with MLL/menin. Other menin mutants (P12L and L22R), however, retained competence to coimmunoprecipitate with MLL but were nevertheless unable to associate with LEDGF (Figures 6A and 6B).

The latter mutants were assessed for their potential to rescue loss of menin function following shRNA knockdown of endogenous murine *Men1* expression in ME-transformed cells (Figure 6C). In contrast to transduced wild-type menin, which prevented loss of clonogenicity caused by *Men1* knockdown, the P12L and L22R mutants failed to maintain myeloid transformation (Figures 6D and 6E). They were also unable to optimally maintain *Hoxa9* expression following inactivation of *Men1* in ME-transformed cells compared with wild-type menin-transduced cells (Figure 6F), consistent with a compromised ability of L22R to maintain *CDKI* transcription in menin-deficient murine embryonic fibroblasts (Milne et al., 2005). Therefore, a subset of menin mutations in MEN1 tumors specifically abrogate LEDGF interaction while preserving MLL interaction but nevertheless compromise MLL-dependent functions. These data support a role for LEDGF in MEN1 tumor suppression as well as MLL-associated leukemogenesis.

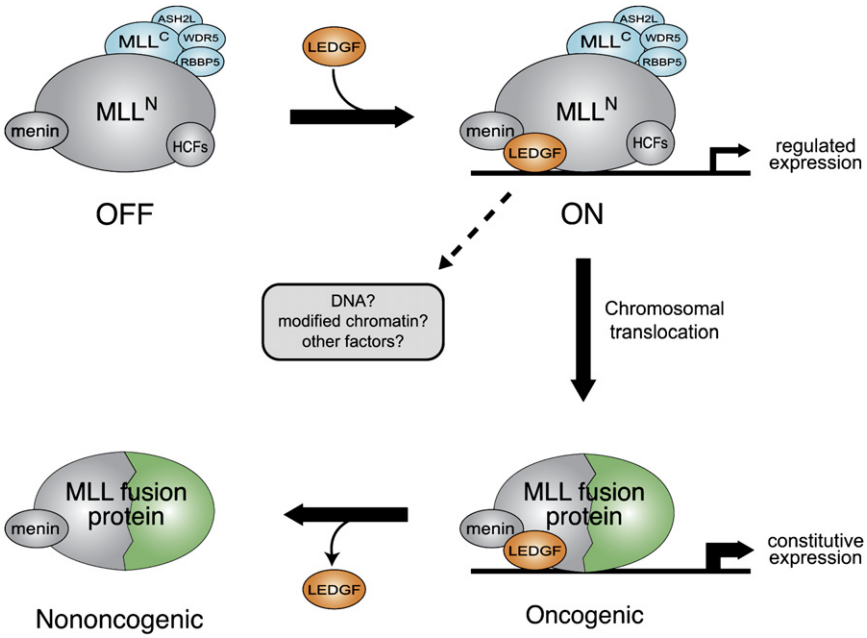

Figure 7. Model for the Role of LEDGF in the Normal and Neoplastic Functions of the MLL/Menin HMT Complex

Top: specific association with LEDGF is required for transcriptional contributions by the MLL/menin HMT complex at its chromatin sites of action. Bottom: similarly, the constitutive transcriptional properties of MLL chimeric oncoproteins in complex with menin are also dependent on association with LEDGF, which may provide a molecular target for therapeutic intervention.

DISCUSSION

Our studies establish LEDGF as a crucial cofactor required for both the oncogenic and tumor suppressor functions of MLL/menin complexes. LEDGF interacts conjointly with MLL and menin on the chromatin of cancer-associated genes to mediate MLL-dependent transcription pathways (Figure 7). In this context, menin serves as an adaptor to link MLL with LEDGF. A subset of menin mutations in MEN1 tumors is particularly informative, as they abrogate interactions with LEDGF, but not MLL, yet compromise MLL/menin activity. Genetic evidence that MLL functions with LEDGF in its normal developmental role to regulate *HOX* gene expression during establishment of the embryonic body plan is suggested by the phenotypic overlaps of *Mll*- and *Ledgf*-deficient mice, which both display skeletal malformations representative of anterior and posterior homeotic transformations (Sutherland et al., 2006; Yu et al., 1995). Thus, LEDGF is an essential component of the MLL/menin HMT complex in the setting of its normal and pathologic activities.

LEDGF has previously been implicated in various transcriptional processes and cellular functions. Originally discovered based on its cofractionation with the general transcriptional coactivator PC4, LEDGF reportedly associates with transcriptional activators and components of the basal transcriptional machinery including RNA pol II subunits (Ge et al., 1998) and contributes to the transcriptional response following environmental stress (Shinohara et al., 2002). Coincidently, menin is also a regulator of stress-induced response in fruit flies, which transcriptionally upregulate expression of various heat-shock proteins following stressful stimuli (Papaconstantinou et al., 2005). Menin has been reported to coimmunoprecipitate with RNA pol II (Hughes et al., 2004), raising the possibility that it links MLL with LEDGF/p75 in higher-order complexes of dynamic composition to regulate specific stages of transcription.

In addition to its transcriptional role, LEDGF is important for lentiviral integration (Ciuffi et al., 2005; Llano et al., 2006; Shun et al., 2007). LEDGF is the dominant binding partner for HIV-1 integrase in human cells (Cherepanov et al., 2003) and tethers it to host chromosomes (Maertens et al., 2003), thereby serving a major role in determining the highly distinctive pattern of lentiviral genome integration within active transcription units (Ciuffi and Bushman, 2006). Thus, physical association of LEDGF with MLL/menin on chromatin may provide a molecular basis for the selective integration of HIV-1 into actively transcribed regions since the epigenetic mark placed by MLL is involved in maintaining chromatin in a state conducive for transcription (Li et al., 2007). A tethering role for LEDGF likely extends to other host proteins as well since the chromosomal association of JPO2, a Myc-interacting protein with transforming activity, is also strictly dependent on LEDGF (Maertens et al., 2006). It remains to be determined whether these various interactions, which target the IBD of LEDGF, are mutually exclusive with MLL/menin and what their implications may be for antilentiviral therapy.

The association of LEDGF with chromatinized DNA is critically dependent on its PWWP domain (Botbol et al., 2008). This highly conserved motif is present in a variety of chromatin-associated proteins involved in transcriptional regulation, DNA repair, and methylation (Stec et al., 2000). It has structural similarities with Tudor, chromo, and MBT domains, all of which are implicated in binding methylated lysine residues on histones (Maurer-Stroh et al., 2003; Li et al., 2007). Structural similarities with the ligand-binding cavities of these evolutionally related domains strongly suggest that the PWWP domain binds to a currently undefined component of chromatin, although a possible role in nonspecific DNA binding has been suggested as well (Lukasik et al., 2006; Nameki et al., 2005; Sue et al., 2004; Qiu et al., 2002). Compelling evidence for the role of LEDGF in targeting the MLL/menin HMT complex to chromatin is provided by the grafting of its PWWP onto the MLL oncoprotein, which was fully capable of bypassing the requirement for menin in oncogenesis, *Hox* gene misregulation, and chromatin association. This artificial construct is structurally similar to the *Arabidopsis thaliana* homologs of MLL (ATX1 and ATX2) (Alvarez-Venegas and Avramova, 2001), which contain PWWP domains in their amino-terminal portions, providing evolutionary support for the functional link between MLL and LEDGF.

Our study indicates significant roles for LEDGF in menin-dependent growth control. Menin has been reported to potentially

interact with DNA (La et al., 2004) and several other proteins (Balogh et al., 2006); however, our data suggest that menin's sole role in MLL leukemia is to recruit LEDGF. Our observation that some MEN1-associated mutations specifically disrupt LEDGF binding and compromise MLL-dependent transcription also implicates LEDGF in MEN1 tumorigenesis. Accumulating evidence indicates that the physiologic growth responses of endocrine lineage cells are heavily dependent on the MLL/menin pathway through regulated expression of CDKIs (Franklin et al., 1998; Milne et al., 2005; Karnik et al., 2005, 2007). In contrast to ATX proteins, the interaction of MLL with LEDGF critically mediated by menin is noncovalent, which provides a potential mechanism for regulating their conditional association. LEDGF is induced upon cellular stress stimuli including serum starvation (Huang et al., 2007) and, interestingly, is secreted from and reenters lens epithelial cells by penetrating the plasma membrane (Singh et al., 1999). Thus, it is tempting to speculate that cell-autonomous or nonautonomous induction of LEDGF may be implicated in the growth control of endocrine and other lineages, perhaps as part of a molecular switch for targeting of the MLL/menin HMT complex to chromatin (Figure 7).

Our results provide a broader context for conceptualizing the various pathologies associated with LEDGF and its binding partners, which appear to be frequently targeted in diverse diseases. In addition to making essential contributions to MLL-mediated leukemias and endocrine tumorigenesis, LEDGF itself is targeted by chromosomal translocations in leukemia that result in its fusion with the nucleoporin NUP98 (Ahuja et al., 2000). The molecular mechanism by which NUP98-LEDGF causes leukemia is unknown; however, our data show that it is capable of associating with MLL/menin, suggesting that it may also perturb the HOX pathway, compatible with the more frequent fusion of NUP98 with HOX proteins themselves in leukemias. Increased LEDGF expression is also a feature of some cancers including acute myeloid leukemia and tumors of breast and bladder origins (Daugaard et al., 2007; Huang et al., 2007), whereas autoantibodies to LEDGF are frequently present in patients with atopic dermatitis and other autoimmune disorders (Ganapathy and Casiano, 2004). The specific roles of MLL/menin in these various diseases merit further investigation, as they may have important implications for therapeutic interventions in cancer, autoimmunity, and AIDS.

EXPERIMENTAL PROCEDURES

Cell Culture and Animal Use

HB1119, ML-2, REH, and U937 cells were cultured in RPMI 1640 medium supplemented with 15% fetal calf serum and nonessential amino acids. 293T, HeLa, and plat-E cells (Morita et al., 2000) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and nonessential amino acids. All experiments on mice in this study were performed with the approval of and in accordance with the Stanford University Administrative Panel on Laboratory Animal Care.

Vector Construction

The pCMV5 FLAG-MLL-ENL vector (Ayton et al., 2004) and pMSCV-neo constructs encoding MLL-ENL and MLL-AF10 (Ayton and Cleary, 2003; DiMartino et al., 2002) were described previously. Expression vectors for various MLL mutants were generated by restriction enzyme digestion and PCR-based mutagenesis. The cDNA for menin was provided by Dr. F. Hosoda. A series of menin mutants tagged with HA were generated by PCR-mediated mutagenesis and cloned into pcDNA3.1/hygro (Invitrogen) or pMSCV-hygro (Clontech). The expression vector for LEDGF was purchased from OriGene Technologies, Inc. The cDNA fragments for p75 and p52 were generated by PCR and cloned into pCMV6. cDNA of Myc-tagged NUP98-LEDGF was generated by PCR using the pMSCV NUP98-HOX9 (Iwasaki et al., 2005) and pCMV6 LEDGF vectors as templates and subsequently cloned into the pcDNA4/HisMax vector (Invitrogen). The shRNA expression vectors were purchased from Open Biosystems (TRCN0000012113 for *Ledgf#1*; TRCN0000012114 for *Ledgf#2*; TRCN000034394 for *Men1*).

One-Step Immunopurification and Identification of MLL/Menin-Associated Proteins

Preparation of nuclear extracts and large-scale immunoprecipitations were performed as described elsewhere (Yokoyama et al., 2004). 293T cells were cultured in ten 175 cm^2 dishes and transfected with pCMV FLAG-MLL-ENL and pcDNA3.1 menin-HA vectors using Lipofectamine 2000 (Invitrogen). Nuclear extracts were prepared from the cells 48 hr after transfection, cleared by ultracentrifugation, and then immunoprecipitated with anti-FLAG M2 agarose beads for 4 hr. After extensive washing, the purified material was subjected to SDS-PAGE analysis and visualized by Coomassie brilliant blue staining. Gel bands containing proteins of interest were subjected to mass spectrometry by the Stanford Proteomic & Integrative Research Facility.

Immunoprecipitation and Immunoblotting

Preparation of nuclear extracts, immunoprecipitation, and immunoblotting were performed as described elsewhere (Yokoyama et al., 2004, 2005). Primary antibodies included mouse monoclonal anti-MLL^N (mmN4.4) and anti-MLL^C (mmC2.1) and rabbit polyclonal anti-MLL^N (rpN1) as described previously (Yokoyama et al., 2002). Rabbit anti-DmMyb was provided by J. Lipsick. Goat anti-menin (C19), mouse anti-Express epitope (Omni-probe D-8), and rabbit anti-SIN3A (K-20) antibodies were purchased from Santa Cruz Biotechnology, Inc. Additional primary antibodies included rabbit anti-menin (BL342) and anti-LEDGF (BL3656) (Bethyl Laboratories, Inc.), mouse anti-LEDGF (BD Transduction Laboratories), and mouse anti-actin (MAB 1501R) (Chemicon). Rat anti-HA antibody (3F10) conjugated with HRP or immobilized to matrix was purchased from Roche. Rabbit anti-FLAG (F-7425) antibody and agarose affinity beads coupled to mouse anti-FLAG M2 or anti-Myc (9E10) monoclonal antibody were purchased from Sigma.

Virus Production

Ecotropic retrovirus was produced using plat-E packaging cells (Morita et al., 2000). Lentivirus was produced by cotransfection of viral vectors with pCMV gag-pol and pVSVG env packaging constructs into 293T cells (Dull et al., 1998). Medium containing virus was collected 48 hr posttransfection and used for transductions.

Myeloid Progenitor Transformation Assay

Myeloid progenitor transformation assays were performed as described elsewhere (Lavau et al., 1997; Yokoyama et al., 2005). Cells (CD45.1) were harvested from the femurs of C57BL/6 or *Men1* floxed ($Men1^{floxed/-}$) mice. Progenitors (c-kit^+) were enriched by immunomagnetic selection (Miltenyi Biotec), transduced with recombinant retroviruses by spinoculation, and plated in methylcellulose medium (M3231, StemCell Technologies, Inc.) containing SCF, IL-3, IL-6, and GM-CSF. In vitro Cre-dependent gene inactivation was performed as described previously (Yokoyama et al., 2005) using 0.1 nM 4-OHT to activate the Cre-ERtam protein. For secondary transductions, 10^5 cells were transduced with retrovirus by spinoculation, cultured in methylcellulose medium overnight, and selected for drug resistance (hygromycin 750 μg/ml or puromycin 4 μg/ml) for at least 2 days.

In Vivo Leukemogenesis Assay

Transformed cells (2×10^5) from methylcellulose cultures were transplanted intravenously into lethally irradiated C57BL/6 mice (900 rads) with 2×10^5 syngeneic bone marrow cells. Moribund mice were sacrificed, and tissues were fixed in 10% formalin and processed for hematoxylin and eosin staining. Cells from bone marrow and spleen were subjected to cytospin preparation followed by May-Grunwald/Giemsa staining or cultured in methylcellulose medium for secondary transplantation.

Quantitative RT-PCR

Reverse transcription and quantitative PCR were performed as described previously using TaqMan probes for *Hoxa9* (Mm00439364_m1), *Men1* (Mm00484963_m1), *Gapdh* (Mm99999915_g1), *Ledgf* (Mm01259222_g1), and β-*actin* (Mm00607939_m1) purchased from Applied Biosystems. Expression levels normalized to those of β-*actin* or *Gapdh* were calculated using a standard curve and the relative quantitation method as described in ABI User Bulletin #2 (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf).

Chromatin Immunoprecipitation

ChIP was performed as described previously (Weinmann and Farnham, 2002; Yokoyama et al., 2005) using primary antibodies specific for MLL^N (rpN1), menin (BL342), LEDGF (BL3656), or histone H3 (ab1791, purchased from Abcam). Semiquantitative or quantitative real-time PCR was performed on the precipitated DNAs using primers and qPCR probes described in the Supplemental Data. The values relative to input were determined using a standard curve and the relative quantitation method as described in ABI User Bulletin #2.

Indirect Immunofluorescence

Indirect immunofluorescence was performed as described elsewhere (Yokoyama et al., 2001) on HeLa cells transfected with expression vectors encoding various MLL mutant proteins. The cells were fixed, incubated with rabbit anti-MLL^N antibody (rpN1), and probed with a FITC-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). Cells were stained with DAPI (Vector Laboratories) and analyzed by confocal immunofluorescence microscopy at the Stanford Cell Sciences Imaging Facility.

SUPPLEMENTAL DATA

The Supplemental Data include four figures and two tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/1/36/DC1/.

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